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# POST-HARVEST ROTS OF TOMATO IN RELATION TO LYASES AND MYCOTOXIN PRODUCTION *IN VITRO* AND *IN VIVO*

S.A. OMAR and A-L.E. MAHMOUD

Department of Botany, Faculty of Science, Assiut University, Assiut, Egypt

ABSTRACT - The post-harvest tomato rotting fungi were isolated from 100 samples of tomato showing rot symptoms. A total of 220 isolates were recovered on Czapek's dextrose agar medium comprising 6 fungal species among which *Alternaria alternata*, *Aspergillus flavus* and *A. niger* were predominant. *Asp. tamarii*, *Cochliobolus spicifer* and *Penicillium citrinum* were isolated at low frequency. Testing enzymatic abilities of some isolates showed that most of these isolates produced cellulase, pectin lyase and polygalacturonase on synthetic media ■ well ■ on inoculated tomatoes. Moreover, some isolates which did not show enzymatic activities on agar media produced enzymes on tomatoes. However, most isolates of *Alt. alternata*, *Asp. flavus* and *Asp. niger* tested were good enzyme producers and showed the highest enzymatic activity either *in vitro* or *in vivo* suggesting that tomato rot is mainly brought about by members of these fungi. It is worthmentioning that the ealthy fruits had no delectable enzymatic activity.

During screening for mycotoxigenicity, *Alternaria alternata* proved to be the most able mould to produce different mycotoxins in liquid medium as well as in infected tomatoes. In addition to *Alternaria* toxins, aflatoxins $B_1$, $B_2$ and citrinin were produced by *Asp. flavus* and *P. citrinum*, respectively. Results of this study clearly showed that tomato fruits infected naturally with the moulds previously mentioned may contain different mycotoxins which may represent a potential health hazard.

RESUMÉ - Les agents fongiques responsables du pourrissement après récolte des tomates ont été isolés de 100 tomates présentant des symptômes de pourrissement. Deux cent vingt souches ont été isolées sur Czapek-Dextrose agar. Ces souches se répartissent parmi six espèces. *Alternaria alternata*, *Aspergillus flavus* et *Aspergillus niger* sont les espèces les plus fréquentes. *Aspergillus tamarii*, *Cochliobolus spicifer* et *Penicillium citrinum* ont été isolés à de faibles fréquences. Des essais enzymatiques réalisés sur certains de ces isolats ont permis de montrer que la plupart produisent des cellulases, des pectine-lyases et des polygalacturonases, aussi bien sur milieu synthétique que sur tomates. Certains isolats ne présentent d'activité enzymatique que sur tomates. La plupart des isolats d'*Alt. alternata*, *Asp. flavus* et *Asp. niger* se sont révélés être de bons producteurs d'enzymes et montrent la plus haute activité, tant *in vitro* qu'*in vivo*, suggérant que le pourrissement des tomates puisse être incriminé à des membres de ces espèces. Les tomates saines ne présentent pas d'activité enzymatique.

Les études mycotoxicologiques ont montré qu'*Alt. alternata* était l'espèce isolée produisant le plus de mycotoxines différentes en milieu liquide ou sur tomates. En plus des toxines produites par *Alt. alternata*, des aflatoxines B1 et B2, et de la citrinine étaient produites respectivement par *Asp. flavus* et *Penicillium citrinum*. Il en résulte que des tomates naturellement contaminées pourraient contenir différentes mycotoxines, représentant un risque pour la santé.

## INTRODUCTION

Tomato fruits represent one of the essential vegetables all over the world throughout the year. After harvesting, these fruits may be invaded by several moulds that probably cause fruit rotting and extensive damage to the crop (Ayres *et al.*, 1964; Barkai-Golan, 1974). Extensive deterioration results in economic loss to commercial marketers of these fruits. Tomato rot is favoured by the high temperature and hence it is pronounced in tropical and subtropical regions (Adisa, 1980) however, some fungi can infect tomatoes stored at 10-12°C causing their spoilage (Ayres *et al.*, 1964).

Infection of fruits by pathogenic fungi is initiated by production of cell wall-degrading and macerating enzymes (Weste, 1970). The role of polysaccharide degrading enzymes in microbial pathogenicity has been reviewed (Wood, 1976). The ability of many pathogenic moulds to produce these enzymes in culture is not sufficient reason to ascribe them a role in pathogenicity (Byrde, 1979).

In addition to biodeterioration of tomato fruits, different mycotoxins may be produced in these fruits by toxigenic moulds and this may constitute a potential health hazard (Harwig *et al.*, 1979; Stinson *et al.*, 1980 and 1981).

The present work was designed to throw some light on the moulds that cause post-harvest spoilage of tomato. Enzymatic abilities and mycotoxin-producing potential of these fungi, both *in vitro* and *in vivo*, were also studied.

## MATERIALS AND METHODS

**Source of samples.** A total of 100 tomatoes showing lesions of different appearance were collected from markets in different localities of Assiut Governorate, Egypt.

**Isolation and identification of moulds.** By using sterile scalpal, tissue fragments were excised from lesions of infected fruits and were plated on Czapek's dextrose agar medium supplemented with rose bengal (65 ppm) as a bacteriostatic agent. Inoculated plates were incubated for 7-10 days at 28°C. The resulting moulds were isolated and identified.

**Enzymatic activity of the isolated fungi.** A total of 40 fungal isolates from fungi recovered during this investigation were screened for their ability to produce some enzymes on solid media as well as on tomato fruits. These fungi were *Alternaria alternata, Aspergillus flavus, A. niger, A. tamarii, Cochliobolus spicifer* and *Penicillium citrinum*.

Cellulase activity was studied by using the method described by Eggins & Pugh (1962). The tested fungi were grown on medium contained (g/L), ammonium sulphate, 0.5; L-asparagine, 0.5; potassium dihydrogen phosphate, 1.0; potassium chloride, 0.5; magnesium sulphate, 0.2; calcium chloride, 0.1; yeast extract, 0.5; cellulose, 10 and agar 20. After 7 days incubation at 28°C, plates were flooded with chloro-iodide of zinc. The uncoloured zone gave a measure of the cellulolytic power of the moulds.

The test isolates were screened on MP-7 and MP-5 media of Hankin *et al.* (1971) for pectin lyase (PL) and polygalacturonase (PG), respectively. After growth of organisms for 7 days at 28°C, pectolytic activities on both media were determined by flooding plates with 7 mol/L HCl solution. This precipitate intact pectin and pectolytic moulds were thus surrounded by clear zones against an opaque medium. The extent of zone of clearing around moulds was used as a measure of the degree of pectolytic activity.

Amylolytic activity of the test fungi were screened according to the methods described in the Society of American Bacteriologists (1957). The experimental medium consisted of 28 g of nutrient agar to which 2 g of soluble starch (Merck) were added per litre. After incubation of the inoculated plates for 7 days at 28°C in darkness, they were flooded with an iodine solution (KI, 15 g and $I_2$ , 3 g litre). A zone void of blue indicated the production of amylase.

Proteolytic activity of moulds was determined using a casein substrate. Each mould culture was inoculated onto the surface of mycological agar (peptone, 10 g; agar, 20 g per litre) to which sterile skim milk (10% solution of powder of defatted milk in water) was added at the rate of 5 ml per 100 ml of medium. After incubation for 7 days at 28°C, complete degradation of milk protein was seen as clearing zone in the somewhat opaque agar around colonies. The extent of the clear zone represented the degree of proteolytic activity.

**Enzymatic activities of the tested fungi on tomatoes.** Each tested mould was inoculated on the surface of tomatoes (individual weight, w = 70-100 g) disinfected with ethanol (90%). The inoculation was done by placing a square block of Czapek's dextrose agar with the fungal spores in two windowshaped wounds per fruit (Vinas *et al.*, 1992). Inoculated fruits were placed in sterile plastic bags and incubated at 28°C for 10 days. Two tomatoes were utilized for each strain.

After incubation period, the decayed tomatoes were taken, blended with 0.9 (v/w) distilled water for 3 min. Fruit extracts were clarified by centrifugation at 15000 x g for 15 min at 4°C. The supernatants were employed as crude enzyme solutions.

Plates containing different solid media specific for detection of amylase, protease, cellulase and pectinases were prepared as previously showed. Under sterilized conditions, 0.5 ml of the enzyme solution was pipetted in a cup made in the center of each plate. After incubation at 28°C for 24 h, the presence of these enzymes was examined.

**Screening for mycotoxigenicity.** Ten isolates of both *Alternaria alternata* and *Aspergillus flavus* and 5 isolates of each of *Asp. niger*, *Asp. tamarii* and *Penicillium citrinum* isolated during this study were screened for mycotoxigenicity on liquid medium as well as on tomatoes.

**Inoculation and incubation procedures.** The tested fungi were grown on yeast extract sucrose medium (YES). Spore suspension of a 7-days old culture of each mould was made and 0.5 ml (approx. $10^6$ spores/ml) was used as an inoculum for each 50 ml quantity of YES medium in 250 ml Erlenmyer flasks. The flasks were incubated

at 28°C for 10 days as stationary cultures under darkness. Two replicates of each strain were analysed.

Like wise, each tested mould was inoculated on the surface of tomatoes. Inoculation and incubation procedures were previously mentioned. Tomatoes were frozen after they had reached the desired stage of rot, as estimated by the extent of external discoloration and kept untill extraction.

**Extraction procedures.** In case of liquid medium, the contents of each flask were homogenized with 50 ml chloroform for 5 min in a high speed blender (16000 rpm). Extraction was repeated three times. The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate, filtered and dried to near dryness on a rotary evaporator. The residue was diluted with chloroform to one ml. The chloroform solution was analysed for the presence of aflatoxins ($B_1$ ,$B_2$ , $G_1$ and $G_2$), patulin and citrinin using thin-layer chromatography.

In case of *Alternaria*, liquid cultures were extracted twice with ethyl acetate (30 ml) by overnight shaking under darkness and filtration. The two extracts were combined, dried over anhydrous sodium sulphate and evaporated to near dryness. The residue was dissolved in 1 ml of methylene chloride and analysed for the presence of alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TeA) on TLC.

The decayed frozen tomatoes were extracted according to Harwig *et al.* (1979). The fruits were thawed, blended with 0.9 (v/w) methanol and 50 ml of n-hexane for 3 min, and then centrifuged at about 2000 rpm for 5 min. for citrinin analysis (*P. citrinum*) , 3 ml of 10 N sulfuric acid was added to the extraction solvent. An aqueous methanol extract was removed by pipette and shaken with two 25-ml portions of chloroform. In case of *Alternaria alternata* analysed for AOH, AME and TeA, 3 ml of 10 N sulfuric acid was added to the aqueous methanol portion. Chloroform extracts were combined and evaporated to near dryness. The residue was dissolved in 1 ml of chloroform and was analysed for the presence of toxins previously mentioned.

**TLC analysis.** This was performed on precoated silica gel plates of kieselegel G type 60 (MERCK) of about 0.3 mm thickness using chloroform-acetone (9-1) as a solvent system. The developed plates were examined under UV light at wave lengths of 254 and 366 nm. Mycotoxins were identified by comparison with appropriate reference standards before and after treatment with p-anisaldehyde and ferric chloride solution as described by Durackova *et al.* (1976). *Alternaria* toxins were analysed according to Harwig *et al.* (1979). Authentic samples of aflatoxins and *Alternaria* toxins were purchased from Sigma Chemical CO., U.S.A. Patulin and Citrinin were obtained from U.S. Department of Agriculture, Northern Research Laboratories, Peoria, Illinois, U.S.A. ,

## RESULTS AND DISCUSSION

During this study, *Alternaria alternata, Aspergillus flavus, Asp. niger, Asp. tamarii, Cochliobolus spicifer* and *Penicillium citrinum* were isolated from deteriorated

tomato fruits. As indicated in Table I, *Alt. alternata* was isolated from 70% of samples followed by *Asp. flavus* (62%) and *Asp. niger* (54%) suggesting the responsibility of these three moulds for biodeterioration of tomatoes. These results confirmed with those of Adebanjo & Shopeju (1993) who isolated *Asp. flavus* and *Asp. niger* at high frequencies from some sundried vegetables over a period of 8 weeks. Also, *Asp. flavus* was reported to be associated with the spoilage of tomato fruits (Fajola, 1979). In a similar study, *Alternaria* spp. were isolated from as many as 51.6% of decaying tomatoes stored at 10-12°C (Ayres *et al.*, 1964). The involvement of *Alt. alternata* in spoilage of stored tomatoes was reported by other workers (Pearson & Hall, 1975). ,

Results presented in Table II revealed that most strains of isolated fungi exhibited cellulolytic, pectolytic, amylolytic and proteolytic abilities when grown on

Table I: Fungi isolated from tomato fruit infected by rotting fungi.

| Fungi isolated | Number of cases of isolation (out of 100 samples) |
|---|---|
| *Alternaria alternata* | 70 |
| *Aspergillus flavus* | 62 |
| *Asp. niger* | 50 |
| *Asp. tamarii* | 15 |
| *Cochliobolus spicifer* | 8 |
| *Penicillium citrinum* | 15 |

Table II: Enzymatic abilities of some moulds, isolated from deteriorated tomatoes, in agar media.

| Organism | Number of tested isolates | Cellulase | Pectin lyase | Polygalac-turonase | Amylase | Protease |
|---|---|---|---|---|---|---|
| *Alt. alternata* | 10 | 5+++<br>3++<br>2-ve | 10++ | 6+++<br>2++<br>2-ve | 5++<br>2+<br>3-ve | 6++<br>2+<br>2-ve |
| *Asp. flavus* | 10 | 8+++<br>2-ve | 8+++<br>2-ve | 5+++<br>3++<br>2+ | 3+++<br>3++<br>2+<br>2-ve | 5++<br>2+<br>3-ve |
| *Asp. niger* | 5 | 5++ | 5++ | 3+++<br>2-ve | 4++<br>1-ve | 3++<br>2-ve |
| *Asp. tamarii* | 5 | 3++<br>2+ | 4+<br>1-ve | 3++<br>1+<br>1-ve | 1++<br>3+<br>1-ve | 3+<br>2-ve |
| *C. spicifer* | 5 | 5++ | 3+<br>2-ve | 4+<br>1-ve | 3+<br>2-ve | 2++<br>2+<br>1-ve |
| *P. citrinum* | 5 | 2++<br>3+ | 5+ | 3+<br>2-ve | 2++<br>2+<br>1-ve | 2++<br>3+ |

+, low activity; +++, high activity; ++, fair activity; -ve, no activity.

synthetic media. On inoculated fruits (Table III), the production of cellulase and pectinases was more pronounced. On the other hand, protease and amylase production was varied since some tested isolates which produced these enzymes on synthetic media did not exihibt any enzymatic activity on tomatoes. In this respect, Adisa (1985) found that cellulase, polymethylgalacturonase and pectinmethyltrans-eliminase were identified *in vivo* and in culture filtrates of two tomato spoilage moulds, *(Asp. flavus* and *Asp. fumigatus*. It was realised that the softening of tissues in ripening peaches was correlated with increase in the pectinic acid content (Shewflet *et al.*, 1971) and increased pectic enzyme activity (Pressey & Avants, 1971). These enzymes bring about the breakdown of polysaccharide components resulting in maceration of tissue and death of host cells. Among cell wall-degrading enzymes, pectinolytic and cellulolytic enzymes

Table III: Enzymatic abilities of some moulds, isolated from deteriorated tomatoes, in inoculated fruits.

| Organism | Number of tested isolates | Cellulase | Pectin lyase | Polygalac-turonase | Amylase | Protease |
|---|---|---|---|---|---|---|
| *Alt. alternata* | 10 | 10+++ | 6++<br>4+ | 7+++<br>3+ | 4+<br>6-ve | 5+<br>5-ve |
| *Asp. flavus* | 10 | 9++<br>1-ve | 8++<br>2+ | 6++<br>4+ | 3+<br>7-ve | 6+<br>4-ve |
| *Asp. niger* | 5 | 5+ | 5+ | 4++<br>1-ve | 2+<br>3-ve | 3+<br>2-ve |
| *Asp. tamarii* | 5 | 3++<br>1+<br>1-ve | 4+<br>1-ve | 4+<br>1-ve | 3+<br>2-ve | 3+<br>2-ve |
| *C. spicifer* | 5 | 5+ | 4+<br>1-ve | 3+<br>2-ve | 2+<br>3-ve | 2+<br>3-ve |
| *P. citrinum* | 5 | 5+ | 5+ | 5+ | 4+<br>1-ve | 4+<br>1ve |

+, low activity; +++, high activity; ++, fair activity; -ve, no activity.

pose a unique position and several references referred the pathogenicity of plant pathogens to the ability of secretion of these enzymes (Weste, 1970; Kachhawaha & Ali, 1982), in spite of the involvement of other enzymes in cell wall degradation. This is because cellulose and pectin represent the main and most complex components of plant cell wall.

During screening for mycotoxigenicity of the tested fungi, 14 out of 35 isolates were toxigenic (Table IV). The toxigenic isolates belonged to *Alternaria alternata* (8 isolates), *A. flavus* and *P. citrinum* (3 isolates for each).

*Alternaria alternata* proved to be the most able species in mycotoxin production where 80% of its tested isolates produced toxins both *in vitro* and *in vivo*. Four isolates produced tenuazonic acid (TeA), 2 isolates produced tenuazonic acid (TeA) in addition to alternariol monomethyl ether (AME) and 2 isolates produced alternariol monomethyl ether (AME) as well as alternariol (AOH). These results suggest that tomato fruits infected naturally with *Alternaria alternata* may contain TeA,

AME and AOH. These compounds are known metabolites of *Alternaria alternata* (Pero *et al.*, 1973).

These results agree with those of Stinson *et al.* (1980) who found that most isolates of *Alternaria* produced TeA, AOH and AME on tomato fruits. They also stated that the known toxigenic strains of *Alternaria* produced more of TeA on tomatoes than the dibenzo-7α-pyrone toxins (AOH and AME). In another study, Stinson *et al.* (1981) reported that TeA was the main mycotoxin produced in *Alternaria*-infected tomatoes from commercial sources while AOH and AME were present in small amounts. Similarly, Harwig *et al.* (1979) found that *Alternaria alternata*, isolated from decayed tomatoes, produced TeA and AME in culture medium as well as in infected tomatoes however, AOH was not detected.

Table IV: Ability of some moulds, isolated from deteriorated tomatoes, to produce mycotoxins in liquid medium and in infected fruits.

| Organism | Number of tested isolates | On liquid medium | | On tomatoes | |
|---|---|---|---|---|---|
| | | Number of toxigenic isolates | Mycotoxins detected | Number of toxigenic isolates | Mycotoxins detected |
| *Alternaria alternata* | 10 | 8 | Altemarial (AOH), Altemariol, Monomethyl ether (AME) & Tenuazonic acid (TeA) | 8 | Altemarial (AOH), Altemariol, Monomethyl ether (AME) & Tenuazonic acid (TeA) |
| *Aspergillus flavus* | 10 | 3 | Aflatoxins B1 & B2 | 3 | Aflatoxins B1 & B2 |
| *Asp. niger* | 5 | - | - | - | - |
| *Asp. tamarii* | 5 | - | - | - | - |
| *Penicillium citrinum* | 5 | 3 | Citrinin | 3 | Citrinin |

From 10 isolates of *Asp. flavus*, one isolate produced aflatoxin B and 2 isolates produced aflatoxins $B_1$ and $B_2$. Published literatures dealing with the production of aflatoxins in infected tomatoes are not available. However, the natural occurrence of aflatoxins in tomato paste samples has been recorded (Saber *et al.*, 1992). In a similar study, Neelakantan *et al.* 1983) found that aflatoxin B was naturally present in apples. Experimental production of aflatoxins in various fruits has been reported (Detroy *et al.*, 1971).

Citrinin was produced by 3 isolates (60%) of *P.citrinum* in YES medium and in tomato fruits. These results agree, to some extent, with those of Harwig *et al.* (1979) who recorded this toxin in culture filtrate as well as in tomatoes inoculated with *P. expansum*.

Results of the present study clearly showed that most of the moulds that cause post-harvest spoilage of tomatoes are enzymatically active. These moulds excrete an array of enzymes which bring about the breakdown of organic matter resulting in

maceration of fruit tissues. In addition to biodeterioration, different mycotoxins may be produced in fruits by toxigenic moulds which represents a potential health hazard.

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